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The effect of an acute bout of exercise on circulating vitamin D metabolite concentrations: a randomised crossover study in healthy adults

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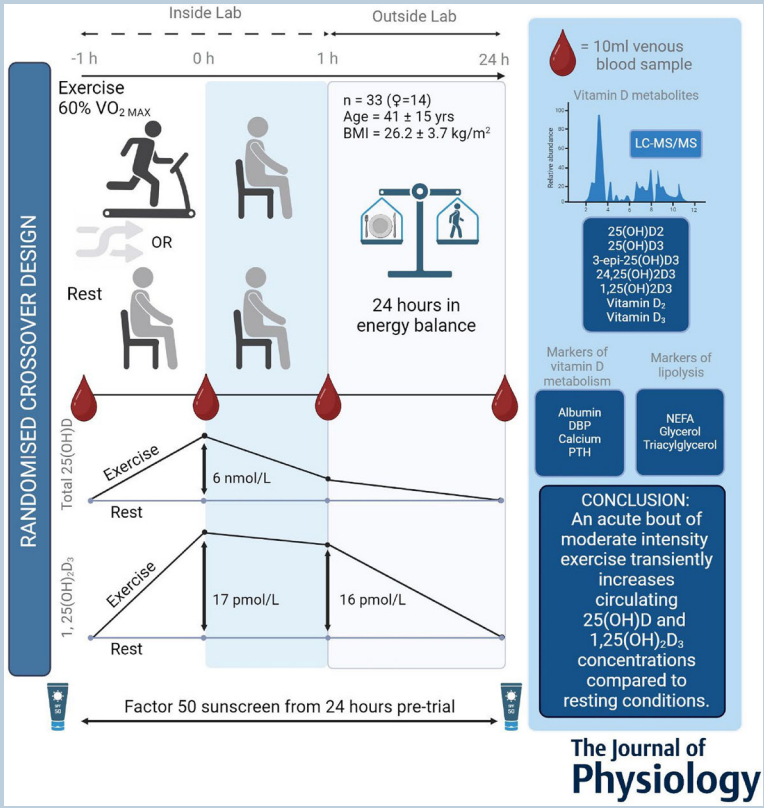

Abstract The effect of acute exercise on circulating concentrations of vitamin D metabolites is unclear. To address this knowledge gap, we examined the effect of a bout of treadmill-based exercise *versus* rest on circulating concentrations of 25(OH)D_3, 25(OH)D_2, 3-epi-25(OH)D_3, 24,25$(OH)_2D_3$, 1,25$(OH)_2D_3$, and vitamin D_2 and D_3 in healthy men and women. Thirty-three healthy adults (14 females, 41 (15) years, body mass index 26.2 (3.7) kg/m^2, $\dot{V}_{O_2max}$ 36.2 (9.2) ml/kg/min; mean (SD))

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completed two laboratory visits involving 60 min of moderate-intensity treadmill exercise (60% $\dot{V}_{O_2max}$) *versus* 60 min of seated rest, both in an overnight fasted-state, as part of a randomised cross-over design. Venous blood samples were drawn at baseline, immediately (0 h), 1 h and 24 h after the exercise or rest-period. There was a significant time × trial interaction effect for total circulating 25(OH)D ($P = 0.0148$), 25(OH)D_3 ($P = 0.0127$) and 1,25$(OH)_2D_3$ ($P = 0.0226$). Immediately post-exercise, 25(OH)D, 25(OH)D_3 and 1,25$(OH)_2D_3$ concentrations were significantly elevated compared to the control resting condition, and 1,25(OH) $_2D_3$ remained significantly elevated 1 h later. Circulating albumin, vitamin D binding protein, calcium and parathyroid hormone were elevated immediately post-exercise. Thus, an acute bout of moderate intensity exercise transiently increases concentrations of circulating 25(OH)D and 1,25$(OH)_2D_3$ compared to resting conditions.

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Abstract figure legend Thirty-three healthy adults (14 ♀, 41 (15) years, body mass index 26.2 (3.7) kg/m^2, $\dot{V}_{O_2max}$ 36.2 (9.2) ml/kg/min; mean (SD)) undertook a randomised crossover trial examining the effect of a 60 min bout of treadmill-based exercise (60% $\dot{V}_{O_2max}$) *versus* seated rest on a range of circulating vitamin D metabolites (as measured by the gold-standard LC–MS/MS), markers related to vitamin D metabolism, and markers of lipolysis. Immediately post-exercise, total serum 25(OH)D and 1,25$(OH)_2D_3$ concentrations were significantly elevated compared to the control resting condition, and 1,25$(OH)_2D_3$ remained significantly elevated 1 h later. Several controls were employed during the trial period: 24 h prior to trials, participants were instructed to apply factor 50 sunscreen and avoid moderate–vigorous physical activity. On trial days, energy balance was achieved by the provision of food to meet estimated energy expenditure, and participants were instructed to avoid moderate–vigorous physical activity during this 24 h period.

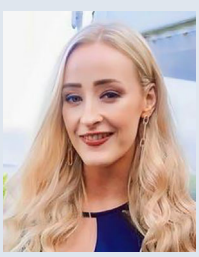
Key points

- Observational studies suggest that acute exercise might change circulating concentrations of vitamin D metabolites, but this has not been investigated using randomised crossover studies and using robust analytical procedures.
- In this study, we used a randomised crossover design to examine the effect of a bout of treadmill-based exercise (*vs.* rest) on circulating concentrations of a wide range of vitamin D metabolites in healthy humans.
- We show that an acute bout of moderate intensity exercise transiently increases concentrations of circulating 25(OH)D and 1,25$(OH)_2D_3$ compared to resting conditions.
- These findings indicate that regular exercise could lead to transient but regular windows of enhanced vitamin D biological action.

Introduction

Several observational studies have shown correlations between engagement in physical exercise and elevations in circulating vitamin D metabolite concentrations for up to 24 h (Barker et al., 2013; Evensen et al., 2019; Maïmoun et al., 2005; Maïmoun et al., 2006; Mieszkowski et al., 2020; O'Leary et al., 2023; Sun et al., 2017;

Following registration as a Dietitian with the Health and Care Professions Council in 2017, **Sophie Davies** received her PhD in 2024 from the University of Bath. Her PhD examined the effect of exercise on vitamin D metabolism and the role of adipose tissue, as part of a wider collaboration between the University of Cambridge and University of Birmingham working on the VitaDEx Study. She is currently working as a specialist dietitian within the NHS and utilising physiological research methods from her PhD in collaboration with the University of Bath, with the aim of improving the quality of nutritional provision amongst inpatients.



Zychowska et al., 2021). Previous research has principally compared the effect of acute exercise on circulating vitamin D metabolites relative to baseline concentrations only. However, pre–post designs cannot account for diurnal rhythm in vitamin D metabolites and their binding proteins (Jones et al., 2017; Rejnmark et al., 2002), and to date no randomised crossover studies with a resting control condition have examined whether this is a causal relationship.

Previous findings are equivocal in terms of the likely circulating 25(OH)D concentration at any given time point following an exercise bout, partly due to variations in the mode, intensity and duration of exercise undertaken, but also due to varying analytical techniques (e.g. enzyme-linked immunosorbent assay (ELISA) *vs*. liquid chromatography–tandem mass spectrometry (LC–MS/MS)). Furthermore, few studies have utilised gold-standard LC–MS/MS to measure circulating vitamin D metabolites beyond 25-hydroxyvitamin D (25(OH)D) following exercise (e.g. 3-epi-25(OH)D_3 and 24,25$(OH)_2D_3$; Mieszkowski et al., 2020; O'Leary et al., 2023; Zychowska et al., 2021). Moreover, several important variables such as diet and vitamin D supplements, or recent physical activity and cutaneous vitamin D synthesis, are frequently unreported or uncontrolled in prior studies examining responses of vitamin D metabolite concentration to acute exercise.

We previously proposed that exercise may release lipid soluble vitamin D into the circulation as a bi-product of lipolysis in adipose tissue (Hengist et al., 2019). Following low- to moderate-intensity exercise, net lipolytic rates remain elevated for up to 24 h (Magkos et al., 2009), and so an exercise-induced increase in net lipolysis could lead to a sustained increase in vitamin D mobilisation from adipose tissue (Hengist et al., 2019). Therefore, the primary aim of this randomised crossover study was to examine the effect of an acute bout of a moderate-intensity treadmill-based exercise at an intensity associated with high rates of lipolysis (i.e. a maximal rate of oxygen uptake ($\dot{V}_{O_2max}$) of 60% for 60 min) on the concentration of circulating 25(OH)D over a period of 24 h. The secondary aims of this study were to examine the response and interaction of other circulating vitamin D metabolites, protein carriers of vitamin D metabolites, and other blood parameters associated with vitamin D metabolism following acute exercise compared to rest.

Methods

Ethical approval

The present study was conducted in accordance with the *Declaration of Helsinki* and was approved by the University of Bath REACH ethics committee in June 2020 (reference number: EP 19/20 026). The study is registered as a Clinical Trial (NCT05214027).

Experimental design

A randomised crossover design was used for this study. Participants undertook two laboratory visits (exercise and rest) in a randomised order allocated by a third party with no involvement in the study via *randomizer.org*. These visits were separated by a period of 7 days (Barker et al., 2013) (mean (SD)) (no less than 3 days) and with a maximum separation of 21 days. Trials involved participants arriving at the laboratory having fasted overnight and refrained from any strenuous activity for 24 h. Following an initial baseline venous blood sample, participants undertook either a 60 min treadmill-based exercise session corresponding to 60% $\dot{V}_{O_2max}$ or a seated rest period for an equal duration. Further venous blood samples were taken immediately on completion of the exercise bout or rest period, after a further 1 h seated rest, and 24 h after the baseline sample. Participants were blinded to trial order until arrival to the laboratory for the first experimental trial. Figure 1 summarises the study design.

Eligibility criteria

Individuals were recruited to the study through word-of-mouth and social media. Data collection was conducted between March 2021 and November 2021 in the South-West of England (University of Bath). Potential participants who expressed an interest in participating were sent written information regarding the study prior to a screening meeting. Participants provided written informed consent prior to commencing the study.

Participants were eligible if they had a body mass index (BMI) between 18.5 and 40 kg/m^2, a stable body mass (<5% total body weight change in past 6 months), and the ability to consent to study procedures.

Individuals were excluded from participating if they self-reported any of the following conditions during screening: coronary heart disease, chronic kidney disease, type 2 diabetes, stroke, heart failure or peripheral arterial disease. Participants were also excluded if they presented with 'severe hypertension' during screening (defined as a blood pressure greater than 180/110 mmHg; British Hypertension Society and NICE guidelines – CG127). Individuals were also excluded if they had regularly taken dietary vitamin D supplements within the last 3 months, used prescribed medication that is known to interfere with lipid metabolism or alter physiological responses to exercise, were smokers, had used sunbeds within the last 3 months, or returned any positive responses to the Physical Activity Readiness Questionnaire (PAR-Q).

Table 1. Participant characteristics

Variable	Data (n = 33)
Age (years)	41 (15)
Height (m)	1.75 (0.81)
Body mass (kg)	79.8 (12.8)
BMI (kg/m^2)	26.2 (3.7)
Waist circumference (cm)	90.5 (12.4)
Hip circumference (cm)	104.7 (10.2)
Waist: hip (ratio)	0.86 (0.1)
RMR (kcal/day)	1657 (320)
$\dot{V}_{O_2max}$ (ml/kg/min)	36.2 (9.2)

Data presented as mean (standard deviation). Abbreviations: BMI, body mass index; RMR, resting metabolic rate.

Participants

Thirty-three healthy participants, males (n = 19) and females (n = 14), completed the study (Table 1).

Preliminary assessments

Resting metabolic rate. Participants arrived at the laboratory during the morning, having fasted overnight and refrained from vigorous intensity physical activity for at least 12 h prior to arrival.

Participants rested for 10 min in a supine position prior to the measurement of resting metabolic rate (RMR) by indirect calorimetry using the Douglas bag method (Compher et al., 2006). A calibrated Servomex 1400 gas analyser was used (Servomex Ltd, Crowborough, UK) and RMR calculated using assumptions of substrate oxidation under resting conditions as described by Frayn (1983), in addition to the adjustment for atmospheric gases (Betts & Thompson, 2012). All testing was conducted at ambient temperatures between 20°C and 25°C.

Maximal oxygen uptake assessment. A maximal treadmill-based exercise test was undertaken to determine $\dot{V}_{O_2max}$ (Lode, Groningen, The Netherlands). Participants wore a heart rate (HR) sensor (Polar, Kempele, Finland) attached to a chest-worn strap with a small amount of ultrasound gel (HealthLife, Peterborough, UK) applied to the strap to enhance signal conduction. The structure of the maximal test was explained thoroughly to the participant, and they were familiarised with the Borg ratings of perceived exertion scale (Borg et al., 1982).

Participants were asked to self-select four increasing speeds where the fourth stage was at a speed that they perceived they would be able to maintain for 1 h. They were given a few minutes prior to the test to familiarise themselves with treadmill speeds. Stages were 3 min in duration and the incline remained at 0% over the first four stages, after which time speed remained constant and

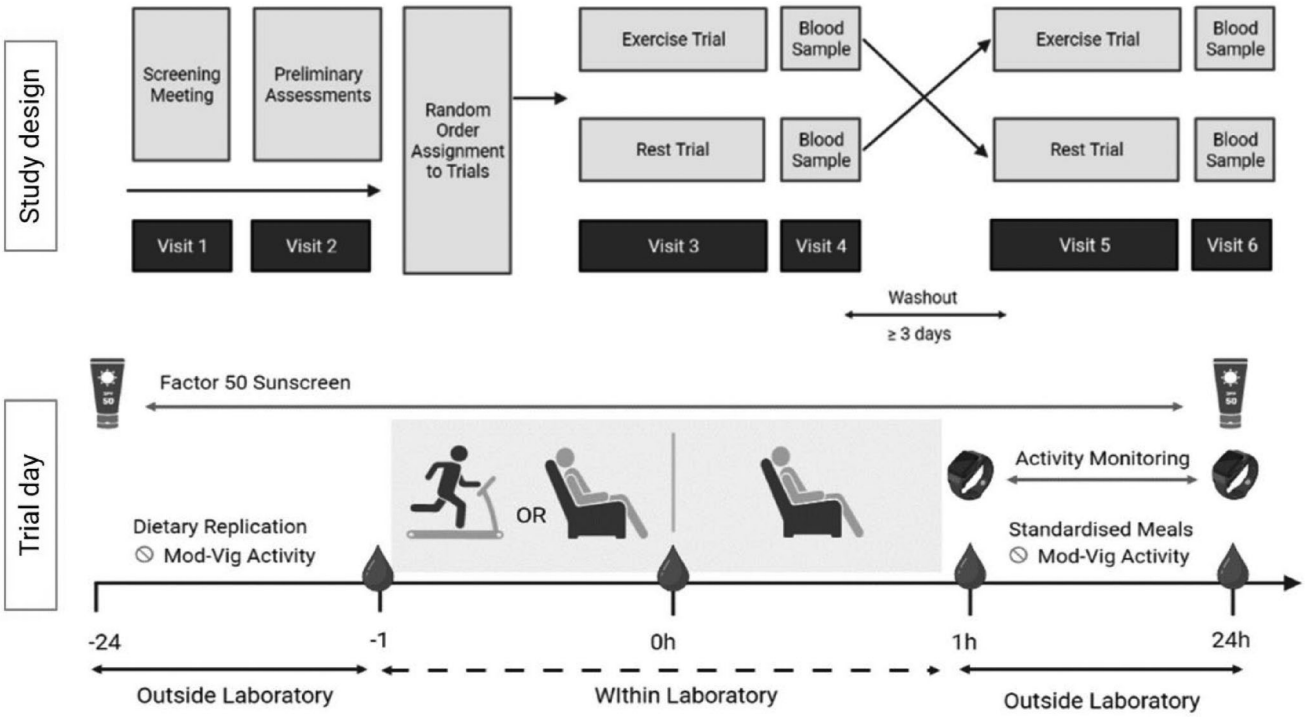


Figure 1. An overview of the trial day and study protocol
Blood samples are 10 ml venous.

incline increased by 3% with each stage until volitional fatigue. Expired air was collected during the last minute of each stage. Heart rate and rating of perceived exertion (RPE) (Borg, 1982) were also noted during this time.

Several criteria were applied to ensure that the end point of the test reflected a valid $\dot{V}_{O_2max}$ value. These included the attainment of age-predicted maximal heart rate (±10 bpm); a Respiratory Exchange Ratio (RER) >1; an RPE of 20; and a $\dot{V}_{O_2}$ plateau (<150 ml change per min) between consecutive time points. Each participant was required to meet at least one of these four criteria (32 participants met two, and two participants met one). For one participant for whom the gas sample collected during maximal exercise stage was lost, a $\dot{V}_{O_2max}$ value was predicted from extrapolating heart rate data against $\dot{V}_{O_2}$.

Experimental trials

Anthropometric measures. Body mass was measured using electronic scales (Tanita, Tokyo, Japan) at the beginning of each visit to the laboratory. Height in the Frankfort plane was measured to the nearest 0.1 cm using a wall-mounted stadiometer (Seca, Hamburg, Germany). Waist and hip circumference measurements were made in triplicate using a non-stretch tape measure (Seca, Hamburg, Germany) and the mean value calculated (WHO, 2008).

Blood sampling. An intravenous cannula was fitted into an antecubital vein to collect 10 ml blood samples throughout the experimental trials, with venepuncture used for the 24 h sample. Blood samples were distributed between an EDTA-coated tube and an untreated serum tube (Sarstedt, Nümbrecht, Germany). The EDTA-coated tube was immediately centrifuged for 10 min (4000 *g*, 4°C), after which the supernatant was immediately removed and stored at −80°C. Serum tubes were left to stand for 30 min at room temperature before being centrifuged and stored as described above.

Exercise bout. The relationships between exercise intensity, $\dot{V}_{O_2}$, HR and RPE from the maximal exercise test during the preliminary assessment were estimated by a linear regression for each participant to calculate the correct treadmill settings to obtain 60% $\dot{V}_{O_2max}$. Participants exercised at this intensity for 60 min on a treadmill. Expired gas samples (1 min) were measured at 15 min intervals and analysed immediately to confirm whether participants were exercising at the correct intensity. If $\dot{V}_{O_2}$ deviated from 60% $\dot{V}_{O_2max}$ by ±5%, treadmill settings were adjusted accordingly. Every 15 min, participants were asked for their RPE, and heart rate was noted. The intravenous cannula was flushed every 15 min with 0.9% sterile NaCl to minimise risk of blockage. Participants were allowed to drink water *ad libitum* throughout both exercise and rest conditions.

Control measures for extraneous variables. Participants who took part in the study between the months of April and September ($n = 27$) were provided with Factor 50 sunscreen (Altruist, Leusden, The Netherlands) to wear from the preliminary assessment until the end of their involvement in the study to limit the cutaneous synthesis of vitamin D during the spring/summer months. Participants were encouraged to apply generously (>2 mg/cm^2) on any exposed areas of skin when spending any time outdoors and to reapply frequently. Participants were also asked about any recent holidays abroad involving increased sun exposure as part of a Health Questionnaire provided at screening, with enrolment delayed by at least 3 months following their holiday.

Physical activity monitoring. To avoid differences in habitual physical activity between experimental trial days, participants avoided any strenuous activity for 24 h after experimental trials. To monitor physical activity on exercise and rest trial days, participants wore a MotionWatch8 (CamNTech, Fenstanton, UK). Epoch length was set to 60 s and a tri-axial recording mode was applied and activity counts recorded.

Provision of food. To maintain energy balance during the 24 h and therefore limit inter-trial differences in total daily net lipolysis, a registered dietitian provided participants with all meals and snacks for the remainder of trial days after leaving the laboratory. Individual 24 h energy requirements were based on an estimated PAL of 1.5 from the measured RMR, as participants were asked to refrain from strenuous activity. For the exercise trial, the additional energy expended during exercise bout was added by increasing portion sizes. Foods known to be natural sources of vitamin D, or those fortified with vitamin D, were avoided in snacks and meals. Participants were asked to return or note down any uneaten food items the following day and match the timing of meals and snacks between trials.

Biochemical analysis. The analysis of vitamin D metabolites was undertaken by researchers blinded to experimental conditions.

Serum vitamin D metabolites. Serum samples at each time point were analysed for vitamin D metabolites 25(OH)D_2, 25(OH)D_3, 1,25(OH)$_2D_3$, 24,25(OH)$_2D_3$, 3-epi-25(OH)D_3, vitamin D_2 and vitamin D_3 by LC–MS/MS using a validated method from Jenkinson et al. (2021) with minor modifications.

Sample preparation – 25(OH)D_2, 25(OH)D_3, 1,25(OH)$_2D_3$, 24,25(OH)$_2D_3$, 3-epi-25(OH)D_3. Vitamin D metabolites were extracted from 300 µl of serum sample combined with 20 µl of internal standard (25(OH)D_3-$^{13}C_5$ instead of 25OHD3-d3) in 1.5 ml microcentrifuge tubes. Samples underwent protein precipitation by the addition of 450 µl isopropanol/water (50/50 v/v) and were vortexed at high speed for 10 min and then left for a further 15 min before centrifuging at 8000 *g* for 5 min. The sample supernatant was transferred to glass tubes for liquid–liquid extraction as described by Kaufmann et al. (2014) with some modifications. The extraction was carried out by the addition of 1 ml hexane for 30 s followed by the addition of 1 ml MTBE, and vortexed for a further 30 s. Samples were frozen at −20°C for 2 h and the resulting organic layer was transferred and evaporated to dryness under nitrogen at 50°C. The dry residue samples were derivatized by the addition of 0.125 mg/ml PTAD (4-phenyl-1,2,4-triazoline-3,5-dione) dissolved in acetonitrile and incubating for 2 h at room temperature in darkness. The reaction was quenched with the addition of 20 µl water and samples were dried under nitrogen and reconstituted in 75 µl water/methanol (50/50 v/v) and then transferred into the well of a 96-well microtitre plate.

Sample preparation – vitamin D_2 and vitamin D_3. Vitamin D metabolites were extracted from 200 µl of serum sample combined with 100 µl of internal standard (trideuterated vitamin D_2 and vitamin D_3) in 12 × 75 mm borosilicate glass tubes. Samples underwent protein precipitation by the addition of 200 µl methanol/water (73/27 v/v) and were vortexed on a multitube vortex mixer for 30 s. The extraction was carried out by the addition of 1.5 ml hexane and vortexing for a further 10 min. Samples were centrifuged for 10 min at 12,300 *g* and the resulting organic layer transferred to clean tubes and evaporated to dryness under vacuum at room temperature. The dry residue samples were derivatized by the addition of 50 µl 0.5 mg/ml PTAD dissolved in acetonitrile. Samples were mixed gently on the multitube vortex mixer for 1 h at room temperature. The reaction was quenched with the addition of 100 µl ethanol and samples were dried under vacuum and reconstituted in 100 µl water–acetonitrile (20/80 v/v) and then transferred into 2 ml amber glass HPLC vials with 150 µl spring bottomed glass inserts. All sample preparation was carried out under yellow light.

LC–MS/MS. For 25(OH)D_2, 25(OH)D_3, 1,25(OH)$_2D_3$, 24,25(OH)$_2D_3$ and 3-epi-25(OH)D_3, analysis was performed on an SCIEX Exion LC system coupled to an SCIEX 6500 QTRAP mass spectrometer (SCIEX, Framingham, MA, USA), using electrospray ionization in positive mode. For vitamin D2 and D3, analysis was performed on a Waters Acquity UPLC system (Waters, Milford, MA, USA) coupled to an SCIEX 5500 QTRAP mass spectrometer, using electrospray ionization in positive mode. For all analytes, the multiple reaction monitoring mode was obtained using settings for the various transitions optimized by infusing pure standard for each analyte into the mass spectrometer. Unit mass resolution was used in both mass-resolving quadruples Q1 and Q3. A single qualifier and another quantifier ion (QI) were optimized for each analyte. For 25(OH)D_2, 25(OH)D_3, 1,25(OH)$_2D_3$, 24,25(OH)$_2D_3$ and 3-epi-25(OH)D_3, the acquisition method was split into three periods during the sample run to quantify groups of metabolites based on retention time: period 1, 0−8.6 min; period 2, 8.6– 16 min; period 3, 16−26.2 min. A Waters UPLC BEH Phenyl (2.1 × 75 mm, 1.7 µm) column was used for liquid chromatography separation of metabolites. The column temperature was set to 40°C and the flow rate was 0.300 ml/min with a mobile phase consisting of A (water–0.1% formic acid) and B (methanol–0.1% formic acid) with the following mobile phase gradient: 0 min: 38% A:62% B; 0.01–12 min: 35% A:65% B; 12.01−22.4 min: 28% A:72% B; 22.41−25 min: 28% A:72% B; 25.01−26.5 min: 38% A:62% B. Two gradient steps were used at 12.01−22.4 and 22.41−25 min to achieve 72% methanol mobile phase composition by 22.4 min and maintain this until 25 min into the sample run. The overall run time was 26.5 min. A 35 µl injection volume was used and the autosampler temperature set to 10°C. For vitamin D_2 and D_3, a Waters Cortecs C18+ (2.2 × 150 mm, 1.6 µm) column was used for liquid chromatography separation of metabolites. The column temperature was set to 45°C and the flow rate was 0.300 ml/min with a mobile phase consisting of A (methanol–0.1% formic acid) and B (water–0.1% formic acid) with the following mobile phase gradient: 0−5 min 80% A; 5.1 min 90% A; 10 min 94% A; 10.1–12 min 100% A to wash out column; 12.1–17 min 80% A for return to starting conditions. The overall run time was 17 min. A 15 µl injection volume was used and the autosampler temperature set to 10°C. The ratio of analyte to internal standard signal was compared to that of a calibration curve to determine analyte concentration.

Clinical chemical analysis. Plasma samples were analysed for lipid metabolites (glycerol, triacylglycerol (TAG), non-esterified fatty acids (NEFA)) and serum samples were analysed for total calcium and albumin using an automated clinical chemistry analyser (Randox Daytona Plus; Randox Laboratories, Crumlin, UK). To eliminate inter-assay variation, each participant's samples for both trials were analysed in the same run.

Vitamin D binding protein and parathyroid hormone. Serum samples at each time point were analysed

for vitamin D binding protein (DBP) using a commercially available ELISA (Immundiagnostik, Bensheim, Germany), and for parathyroid hormone (PTH) using a Roche Cobas analyser (electro-chemiluminescence sandwich assay) according to manufacturer's instructions.

Calculations

Total 25(OH)D. Total 25(OH)D was calculated using the following equation:

$$\text{Total 25 (OH) D} = 25\,(\text{OH})\,\text{D}_2 + 25\,(\text{OH})\,\text{D}_3$$

Free 25(OH)D. Free 25(OH)D was calculated using (Bikle et al., 1986):

$$\text{Free 25 (OH) D} = \frac{\text{total 25 (OH) D}}{1 + \left(6 \times 10^5 \times \text{albumin}\right) + \left(7 \times 10^8 \times \text{DBP}\right)}$$

where free 25(OH)D is concentration of free 25(OH) vitamin D in mol/l; K_{alb} is affinity constant between 25(OH) vitamin D and albumin in mol^{-1} (6×10^5); K_{DBP} is affinity constant between 25(OH)D vitamin D and DBP in mol^{-1} (7×10^8); albumin is concentration of total serum albumin in mol/l; DBP is concentration of total vitamin D-binding protein in mol/l (assuming a MW of 58,000 g/mol); and total 25(OH) vitamin D is concentration of total 25(OH)D in mol/l.

Correcting for haemoconcentration. As described previously, serum total calcium concentrations were used to correct vitamin D metabolites and associated markers for changes in haemoconcentration due to exercise (Alis et al., 2015; Stunes et al., 2022; Sun et al., 2017). The formulas used to estimate haemoconcentration were (Alis et al., 2015):

$$\Delta\ (\%) = 100 \times ((\text{Calcium}_{\text{post}} - \text{Calcium}_{\text{pre}})/\text{Calcium}_{\text{pre}})$$

and

$$\left[\text{parameter}\right]_c = \left[\text{parameter}\right]_u \times (1 + \Delta\,(\%)\,/100)$$

Where c is corrected and u is uncorrected.

Sample size estimation. Sample size was estimated using G*Power 3.1 software and previously published observational data for the mean/SD 25(OH)D concentrations after exercise (Sun et al., 2017). In this prior study, mean (SD) serum 25(OH)D for pre- and 24 h post-exercise measures was 69 (25.7) nmol/l and 74.8 (28.5) nmol/l, respectively (Sun et al., 2017). Based on the G*Power calculated effect size ($dz = 0.71$), a two-tailed repeated measures design with 18 participants would provide 80% probability (power) to detect such an effect at an α-level of $\leq$0.05. Given that the inclusion criteria in the present study were broader than that of Sun et al (2017) (e.g. age and body composition), a smaller effect size ($d = 0.5$) was anticipated, with a recruitment target of 34 participants.

Statistical analysis and data handling. Data were analysed using GraphPad Prism version 9.5.0 (GraphPad Software, Boston, MA, USA) for Windows. Significance was accepted at $P \leq 0.05$. All data are presented as means (SD) in text and tables and means [95% confidence interval (CI)] when presented in figures. Normality was assessed using the Shapiro–Wilks test of normality, and $\log_{10}$ transformed if not sufficiently normally distributed. Two-way repeated measures ANOVA was used to examine the response of serum vitamin D metabolites to exercise or rest over a 24 h period. When time × trial interactions were significant ($P \leq 0.05$), a Bonferroni adjusted t test was undertaken. Cohen's d effect sizes with bootstrapped 95% CIs for the difference between conditions at a given time point were calculated using the EstimationStats.com web app (Ho et al., 2019). Small, moderate and large effect sizes were taken as 0.2, 0.5 and 0.8 respectively. If individual data for specific time points were missing (e.g. missing blood sample), the participants' baseline sample for that respective trial was multiplied by the group average percentage change from baseline to the respective time point. There were no data missing across exercise trials, and five samples missing across rest trials (3× at the 24 h time point, 1× at the 1 h and 1× at the 0 h time point). Two total serum calcium samples were replaced with a mean value for that time point due to unphysiological values. The samples for one participant were inadvertently destroyed, which gave a final sample of $n = 33$. Data were analysed to examine whether there were order effects irrespective of condition and there was none.

Results

Serum vitamin D metabolites

Table 2 summarises the effect of exercise and rest on circulating vitamin D metabolites and associated markers. A two-way repeated measures ANOVA showed a significant time × trial interaction for total circulating 25(OH)D ($P = 0.0148$; Fig. 2*A*), $25(OH)D_3$ ($P = 0.0127$; Fig. 2*C*) and $1,25(OH)_2D_3$ ($P = 0.0226$; Fig. 2*E*). There were no significant time × trial interaction effects for circulating $24,25(OH)_2D_3$, 3-epi-$25(OH)D_3$ and vitamin D_3. Circulating vitamin D_2 was only detected in samples from four participants and is therefore not presented.

Where interaction effects were observed, Bonferroni-adjusted *post hoc* analysis (t tests) identified greater concentrations in the exercise trial at the 0 h time

point for total circulating 25(OH)D (Fig. 2*A*), circulating 25(OH)D_3 (Fig. 2*C*) and serum 1,25$(OH)_2D_3$ (Fig. 2*E*), and also at the 1 h time point for circulating 1,25$(OH)_2D_3$. The magnitude of the difference at 0 h for exercise and resting conditions was 6.04 (95% CI: 2.57–9.51) nmol/l ($P < 0.001$; $d = 0.24$, 95% CI: 0.12–0.36) for total circulating 25(OH)D; 5.58 (95% CI: 2.31–8.85) nmol/l ($P < 0.001$; $d = 0.23$, 95% CI: 0.11–0.35) for circulating 25(OH)D_3; and 17.48 (95% CI: 6.05–28.91) pmol/l ($P = 0.00122$; $d = 0.41$, 95% CI: 0.20, 0.64) for circulating 1,25$(OH)_2D_3$. The magnitude of difference at the 1 h time point for circulating 1,25$(OH)_2D_3$ was 15.56 (95% CI: 4.54–26.58) pmol/l ($P = 0.00291$; $d = 0.19$, 95% CI: 0.02–0.36).

The individual change from −1 h to 0 h, with mean and 95% CI, for total circulating 25(OH)D, 25(OH)D_3, and 1,25$(OH)_2D_3$ are displayed in Fig. 2*B*, *D* and *F* respectively. The difference in change scores for total 25(OH)D was 4.53 (95% CI: 0.43–8.63) nmol/l ($P = 0.0314$; $d = 0.59$, 95% CI: 0.06–1.13), for 25(OH)D_3 was 4.34 (95% CI: 0.46–8.31) nmol/l ($P = 0.0295$; $d = 0.60$, 95% CI: 0.05–1.13), and for 1,25$(OH)_2D_3$ was 12.58 (95%

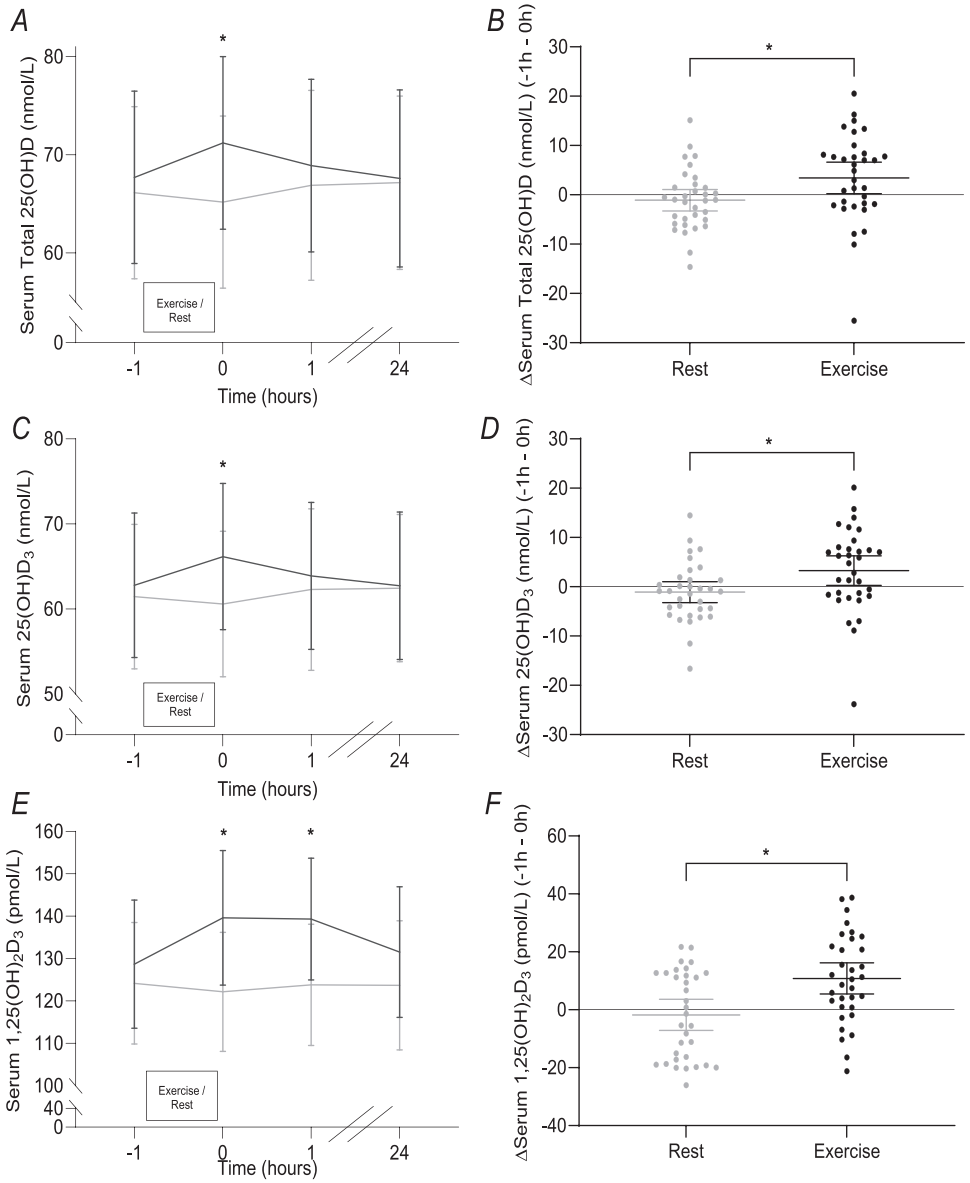


Figure 2. The effect of exercise and rest on total circulating 25(OH)D (*A* and *B*), circulating 25(OH)D_3 (*C* and *D*) and circulating 1,25$(OH)_2D_3$ (*E* and *F*) ($n = 33$)
Dark grey line, exercise; light grey line, rest. The mean and 95% CI data are shown for exercise and rest (*A*, *C* and *E*), where * denotes $P < 0.05$ (Bonferroni correction). The mean and 95% CI and individual data are shown for changes in exercise *versus* rest from pre to the 0 h post time point (*B*, *D* and *F*), where * denotes $P < 0.05$ (paired *t* test).

Table 2. Circulating parameters related to vitamin D metabolism

	Pre		0 h		1 h		24 h		Time × trial interaction
Circulating parameter	Rest	Exercise	Rest	Exercise	Rest	Exercise	Rest	Exercise	*P*-value
25(OH)D_2 (nmol/l)	4.7 (2.8)	4.9 (3.1)	4.6 (2.7)	5.1 (3.1)	4.6 (2.6)	4.9 (2.9)	4.6 (2.7)	4.8 (3.1)	0.183
Free 25(OH)D (pmol/l)	14.1 (5.9)	14.1 (5.4)	14.7 (6.3)	14.1 (5.4)	14.7 (6.6)	14.5 (6.0)	13.9 (5.6)	14.3 (5.8)	0.480
3-epi-25(OH)D_3 (nmol/l)	3.2 (1.8)	3.2 (1.6)	3.1 (1.7)	3.3 (1.7)	3.2 (1.8)	3.2 (1.7)	3.2 (3.3)	3.2 (1.7)	*0.083*
24,25(OH)$_2D_3$ (nmol/l)	5.0 (2.7)	5.1 (2.7)	4.9 (2.4)	5.2 (2.7)	5.0 (2.7)	5.2 (2.8)	5.0 (2.6)	5.1 (2.7)	0.179
Vitamin D_3 (nmol/l)	3.3 (4.7)	4.3 (7.9)	3.1 (4.3)	4.5 (8.4)	3.2 (4.7)	4.2 (7.7)	3.1 (4.4)	4.0 (7.5)	0.172
DBP (mg/l)	371.8 (83.4)	375.9 (65.7)	365.1 (74.6)	407.6* (98.6)	375.1 (86.6)	372.8 (108.5)	377.8 (97.5)	371.7 (78.9)	**0.040**
Albumin (g/l)	43.7 (2.4)	44.1 (2.8)	42.7 (2.5)	46.8* (3.3)	43.8 (3.0)	45.2 (3.6)	44.3 (2.9)	45.1 (2.5)	**<0.001**
PTH (pmol/l)	3.4 (0.88)	3.4 (1.26)	2.9 (0.80)	4.3* (1.87)	3.1 (1.06)	2.9 (0.9)	3.2 (1.3)	3.1 (0.8)	**<0.001**
Total calcium (mmol/l)	2.27 (0.12)	2.28 (0.12)	2.25 (0.12)	2.33* (0.13)	2.27 (0.11)	2.31 (0.13)	2.27 (0.10)	2.27 (0.09)	**<0.001**

Data expressed as means (SD). Bold values indicate significance ($P < 0.05$). *Significant difference between conditions at the same time point, as determined by Bonferroni-adjusted *t* test.

CI: 4.31–20.85) pmol/l ($P = 0.00400$; $d = 0.83$, 95% CI: 0.29–1.38).

Associated markers of vitamin D metabolism

A two-way repeated measures ANOVA showed significant time × trial interactions for circulating albumin, DBP, calcium and PTH (Table 2), with Bonferroni-corrected *t* tests indicating differences between exercise and resting conditions for all these parameters at 0 h.

Exercise bout responses

Participants completed 60 min of continuous treadmill-based exercise at 6.6 (1.4) km/h at an incline of 2.2 (2.6)%. The exercise elicited a mean oxygen consumption equivalent to 61 (3)% $\dot{V}_{O_2max}$, a total energy expenditure of 504 (141) kcal/h and a total lipid oxidation of 21 (10) g/h. The mean RER was 0.88 (0.06). Mean heart rate was 131 (15) beats/min, and the average RPE was 12 (2).

Each sample was analysed for markers of lipolysis (circulating NEFA, glycerol, TAG). A two-way ANOVA showed a significant time × trial interaction between exercise and resting trials for NEFA ($P < 0.001$), glycerol ($P < 0.001$) and TAG ($P < 0.001$) (Fig. 3). Bonferroni-adjusted *post hoc* analysis (*t* tests) indicates that circulating NEFA, glycerol and TAG were all higher at 0 h in the exercise trial (Fig. 3).

The differences between exercise and rest trials at the 0 h time point for circulating NEFA was 0.55 (95% CI: 0.33–0.77) mmol/l ($P < 0.001$); glycerol: 0.19 (95% CI: 0.11–0.26) mmol/l ($P < 0.001$); and TAG: 0.30 (95% CI: 0.12–0.49) mmol/l ($P < 0.001$). There was also a significant difference between exercise and rest trials at the 1 h time point for circulating NEFA (0.13 (95% CI: 0.33–0.77) mmol/l; $P = 0.0185$). The individual change from −1 h to 0 h, with mean and 95% CI, for total circulating NEFA, glycerol, and TAG are displayed in Fig. 3*B*, *D*, and *F*, respectively. The difference in change scores for NEFA was 0.41 (95% CI: 0.34–0.47) mmol/l ($P < 0.0001$), for glycerol was 0.1 (95% CI: 0.13–0.25) mmol/l ($P < 0.0001$), and for TAG was 0.15 (95% CI: 0.07–0.23) mmol/l ($P < 0.001$).

Physical activity

There was no significant difference in physical activity during the 22 h between the 1 h time point and 24 h time point, based on triaxial counts derived from MotionWatch8 devices between trials (exercise trial (3.79 (1.62) × 10^5) and rest trial (3.65 (1.10) × 10^5; $P = 0.617$). Activity monitoring data from three participants were unavailable due to water damage to one

device, and insufficient availability of devices at one point of testing ($n = 2$).

Discussion

This is the first research to examine the causal effects of acute exercise on circulating vitamin D metabolites. We demonstrate that a single bout of exercise significantly increased circulating 25(OH)D and $1,25(OH)_2D_3$ concentrations compared to seated rest. The response of circulating 25(OH)D to exercise was transient, with concentrations of 25(OH)D being no different from resting conditions 1 h or 24 h after exercise. In contrast, circulating $1,25(OH)_2D_3$ remained significantly elevated 1 h following exercise but was not different from resting conditions at the 24 h time point. These findings raise a number of interesting questions regarding flux of vitamin D metabolites between tissues and compartments, as well as whether repeated transient exercise-induced increases in vitamin D metabolite concentrations might meaningfully contribute to overall vitamin D biological action and status.

At the 0 h time point, there was a 6 nmol/l difference between circulating 25(OH)D following exercise and resting conditions. This effect was transient and there were no differences in any vitamin D metabolites between trials

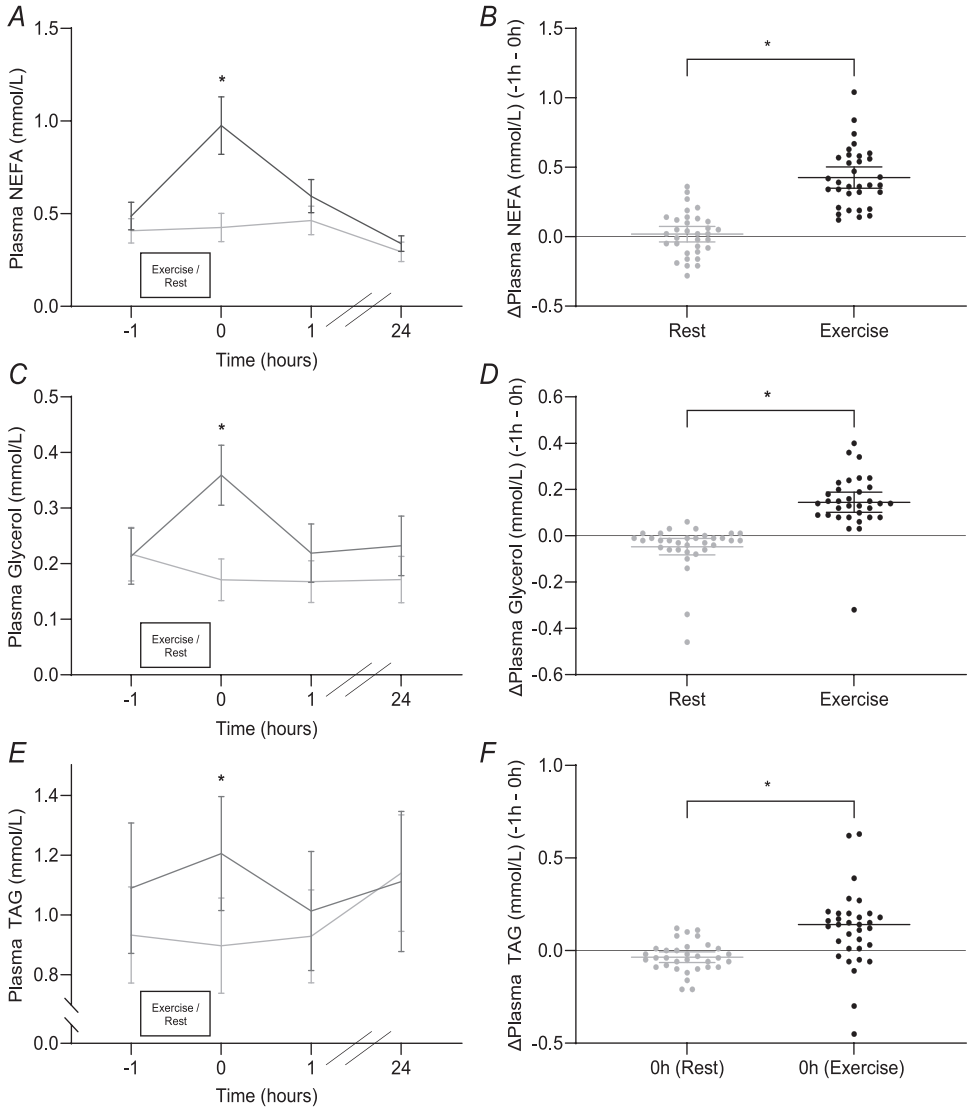


Figure 3. The effect of exercise and rest on plasma NEFA (*A* and *B*), plasma glycerol (*C* and *D*) and plasma TAG (*E* and *F*) ($n = 33$)
Dark grey line, exercise; light grey line, rest. The mean and 95% CI data are shown for exercise and rest (*A*, *C* and *E*), where * denotes $P < 0.05$ (Bonferroni correction). The mean and 95% CI and individual data are shown for changes in exercise *versus* rest from pre to the 0 h post time point (*B*, *D* and *F*), where * denotes $P < 0.05$ (paired *t* test).

after 24 h. One explanation for the return of circulating 25(OH)D back to baseline 1 h post-exercise may be due to the conversion of 25(OH)D to $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$. However, whilst this may also possibly explain the more prolonged elevation of $1,25(OH)_2D_3$, this does not explain the lack of increase in circulating $24,25(OH)_2D_3$. Alternatively, there may be (re)uptake of circulating 25(OH)D into tissues (i.e. adipose or muscle) following exercise. Thus, the present study provides robust evidence demonstrating that moderate-intensity exercise has the capacity to change some vitamin D metabolites acutely, including the conventional measure of vitamin D 'status' (i.e. 25(OH)D) and the bioactive form $1,25(OH)_2D_3$. However, other metabolite concentrations, namely $25(OH)D_2$ 3-epi-$25(OH)D_3$, $24,25(OH)_2D_3$ and calculated free 25(OH)D, were not changed by exercise.

This study demonstrates that relatively modest exercise can transiently increase circulating 25(OH)D. Other studies employing observational time-series ('pre-to-post') designs have reported larger and more sustained changes in response to more demanding exercise bouts in well trained individuals. For example, vitamin D non-supplementing male participants who completed an ultra-marathon showed a substantial increase of ∼35 nmol/l (∼60%) $25(OH)D_3$ 24 h after the race (Mieszkowski et al., 2020). Furthermore, males and females who undertook a 36 h military field exercise showed an average increase in $25(OH)D_3$ of 7.2 nmol/l (10%) and 11.7 nmol/l (16%), respectively, from baseline to the recovery phase 72 h post-exercise (O'Leary et al., 2023). Although these large changes in circulating 25(OH)D cannot be interpreted as causal given the absence of a resting control condition, and these studies did not control for exercise-induced haemoconcentration, they may nonetheless provide speculative support for the notion that there is a dose-response for exercise-induced changes to 25(OH)D. This is broadly consistent with the notion that the post-exercise increase in 25(OH)D could be caused by the mobilisation of vitamin D from adipose tissue. The total energy cost of undertaking an ultra-marathon has been estimated to be 7736–15,367 kcal (Rontoyannis et al., 1989), and that of a 36 h intensive army training protocol between 2000 and 3000 kcal/day (O'Leary et al., 2023). Such prolonged and demanding exercise would promote a significant energy deficit and a requirement for enhanced lipid mobilisation during and after exercise, which could, in parallel, lead to more substantial release of vitamin D_3 and/or 25(OH)D from adipose tissue. This is analogous to the dose response between the increase in circulating 25(OH)D and the degree of weight loss (Mason et al., 2011). In the present study, the total energy demand was ∼500 kcal and estimated lipid oxidation during exercise was ∼20 g. Thus, it appears as though there may be a dose response between the energy demand of the exercise and the resultant change in 25(OH)D.

Whilst the mobilisation of lipid soluble vitamin D from adipose tissue is a potential explanation for an exercise-induced increase in 25(OH)D, there are alternative explanations to consider. For example, the effect of acute exercise on hepatic vitamin D metabolism may contribute to the effects observed in this study (Hoene & Weigert, 2010; Trefts et al., 2015). Furthermore, there is also emerging evidence to demonstrate the role of skeletal muscle as an extravascular storage site of vitamin D (Makanae et al., 2015). It is hypothesised that the uptake of 25(OH)D and binding with DBP within the skeletal muscle acts as a protective storage mechanism against catabolic pathways, mitigating vitamin D insufficiency by prolonging the half-life of 25(OH)D (Mason et al., 2019). Once intra-muscular DBP is proteolysed, it is suggested that 25(OH)D is then released back into the circulation, and this could represent an alternative explanation for the acute increase in 25(OH)D. Abboud et al. (2017) propose a mechanism whereby PTH enhances the uptake of DBP but also stimulates the breakdown of DBP–actin complexes in muscle (Abboud et al., 2017). However, it currently remains unclear to what extent (if any) acute resistance exercise provides a stimulus for proteolysis of the DBP–actin complex in muscle cells. Studies which have employed resistance exercise (and therefore have relatively low lipid utilisation) have reported acute increases in serum 25(OH)D of ∼6 nmol/l (∼25% increase from baseline) immediately following an intense-stretch shortening contraction protocol (Barker et al., 2013), and 11.4 nmol/l (∼15% increase from baseline) immediately following a leg press protocol ($4 \times 8-10$ repetitions at 80% of 1 repetition max) (Evensen et al., 2019). In addition to the increase in circulating 25(OH)D concentration, we also observed a similar transient increase in DBP concentration. Thus, an alternative or parallel explanation for the acute increase in 25(OH)D observed in the current study is release from contracting skeletal muscle and/or changes in hepatic vitamin D metabolism.

This is the first study to show a rise of circulating $1,25(OH)_2D_3$ after an acute bout of exercise. We observed a difference of 17.5 pmol/l between resting and exercise conditions at the 0 h time point, with this difference remaining apparent (15.56 pmol/l) at the 1 h time point. $1,25(OH)_2D_3$ is the biologically active form of vitamin D with a half-life of 4−6 h (Holick, 2009). This raises the intriguing possibility that, if acute exercise was repeated regularly, this could lead to transient but regular windows of enhanced vitamin D biological action. The exercise-induced increase in $1,25(OH)_2D_3$ may be due to the activation of its precursor, $25(OH)D_3$, which was transiently increased immediately following exercise, or the influence of exercise on circulating

PTH and calcium. The PTH–calcium–1,25(OH)$_2$D axis is extremely dynamic, with prior acute exercise studies showing that a rise in PTH stimulates the production of 1,25(OH)$_2$D, and a decrease in serum calcium increases PTH within seconds (Kohrt et al., 2018; Townsend et al., 2016). However, current reporting of 1,25(OH)$_2$D$_3$ and its regulators (i.e. PTH, calcium) following acute exercise remains scarce, and there is also inconsistent sampling and discrepancies between measurement of calcium fractions and terminology used (i.e. total calcium, albumin-adjusted calcium and ionised calcium). O'Leary et al. (2023) observed no change in albumin-adjusted calcium concentrations immediately following a 36 h military style field exercise with a transient increase in PTH (O'Leary et al., 2023), whereas Sun et al. (2017) observed an elevation in total calcium immediately following 30 min of cycling at 70% $\dot{V}_{O_2max}$, which returned to baseline levels within 30 min along with a transient increase in PTH (Sun et al., 2017). Sun et al. (2017) speculated that the lack of increase observed in circulating 1,25(OH)$_2$D following 30 min of cycling at 70% $\dot{V}_{O_2max}$ is likely due to 25(OH)D being converted to 1,25(OH)$_2$D within local tissues (Sun et al., 2017). It is not possible to confirm whether and to what extent tissue conversion may have occurred within the present study, although the conversion of 25(OH)D does not appear limited to local tissues. Furthermore, Sun et al. (2017) did not utilise mass spectrometry to determine 1,25(OH)$_2$D$_3$ and therefore different methods and differences in analytical precision may explain the contrasting effects observed, as well as differences in exercise intensity, duration and study participants. Given the potential physiological importance of exercise-induced changes in biologically active 1,25(OH)$_2$D, further research is required in different modes, intensities and durations of acute exercise.

A particular strength of this study is its randomised crossover design, which examines the effect of both exercise and resting conditions on a range of serum vitamin D metabolites, with each participant serving as their own control to minimise confounding variables. This robust experimental design has not previously been employed to address this research question. Furthermore, several rigorous control measures were employed to avoid the potential confounding effects of variations in lipolysis. Food was provided during trial days to meet estimated energy expenditure (with the additional energy cost of exercise factored in), in addition to the standardisation of dietary intake 24 h prior to trial days. To aid maintenance of energy balance during trials, participants refrained from moderate–vigorous activity 24 h prior to trials and during, with activity monitors were worn during trial days until the 24 h blood sample. Participants also wore factor 50 sunscreen during summer months (March–October) and were instructed to cover up as much as possible whilst participating in the study to minimise any confounding cutaneous vitamin D synthesis. This is the first study to measure a wide range of serum vitamin D metabolites (including vitamin D$_2$ and D$_3$, which have not previously been examined in the context of exercise) via the gold-standard of mass spectrometry following acute exercise in participants of both sexes with a wide range of characteristics (i.e. cardiorespiratory fitness, age and BMI). Finally, the exercise examined in this study was selected to maximise the rate of lipolysis and therefore potential vitamin D release from adipose tissue within a duration of exercise that is realistic and feasible for much of the general population. A limitation to the study is that we do not have regular measurements in the post-exercise period, and thus we do not know at what time point post-exercise changes in circulating 1,25(OH)$_2$D$_3$ returned to baseline. Also, we used calcium to correct for haemoconcentration as used in several exercise studies (Alis et al., 2015; Stunes et al., 2022; Sun et al., 2017), but we cannot compare this to estimation of haemoconcentration correction using the more common Dill and Costill method (Dill & Costill, 1974).

To conclude, this study demonstrates for the first time that 1 h of moderate-intensity exercise increases circulating 25(OH)D and 1,25(OH)$_2$D$_3$ concentrations transiently compared to resting conditions of equal duration in healthy men and women with a broad range of age, BMI and fitness. Future research should aim to explore the long-term and downstream effects that repeated regular exercise has on vitamin D metabolism, including the examination of vitamin D metabolite concentrations in specific tissues and compartments (i.e. adipose and muscle tissue).

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Additional information

Data availability statement

The data is available at: https://doi.org/10.15125/BATH-01406. For the purpose of open access, the author has applied a creative commons attribution (CC BY) licence to any author accepted manuscript version arising.

Competing interests

All authors have no conflicts of interest to declare.

Author contributions

S.E.D., O.J.P., J.A.B., J.T.G. and D.T. designed the research. S.E.D. and O.J.P. undertook preliminary pilot testing of the research methods. S.E.D. undertook recruitment and screening of participants, conducted the research, and analysed biochemical samples for peripheral markers of vitamin D metabolism and lipolysis. M.H. coordinated and C.J. analysed samples for serum vitamin D metabolites. K.S.J. coordinated and S.R.M. and D.A.P. analysed samples for serum DBP, vitamin D_2 and D_3. S.E.D. analysed the data, performed statistical analysis and wrote the initial draft of the manuscript. All authors developed the subsequent drafts of the manuscript and approve the final version. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Keywords

25(OH)D, $1,25(OH)_2D_3$, calcitriol, lipolysis, metabolism, physiology

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

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